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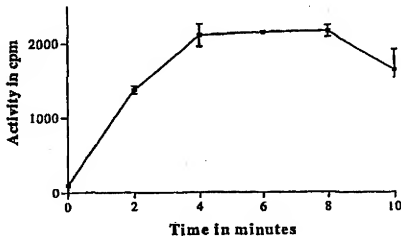
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(54) Title: ASSAY FOR DETECTING PHOSPHO-N-ACETYLMURAMYL-PENTAPEPTIDE TRANSLOCASE ACTIVITY



(57) Abstract

The invention provides an assay for detecting phospho-N-acetylmuramyl-pentapeptide translocase enzyme activity, which comprises the steps of: (1) incubating a reaction mixture comprising, in aqueous medium, N-succinimidyl [2,3-³H]propionate substituted UDP-MurNAc-pentapeptide, N-succinimidyl propionate substituted UDP-MurNAc-pentapeptide (non-radioactive), a source of divalent metal ions, a source of undecapryl phosphate, a source of translocase enzyme and a detergent, under conditions suitable for enzyme activity to occur; (2) acidification of the reaction mixture with a suitable buffer comprising a quaternary ammonium salt at pH ~4.2 to stop the enzyme reaction of step (1); and (3) extraction of any undecaprenol-pyrophosphate-[2,3-³H]propionate-N-acetylmuramyl-pentapeptide product formed and measuring radioactivity using a scintillation counter; and also a kit for use therein.

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Assay for detecting phospho-*N*-acetylmuramyl-
pentapeptide translocase activity

The present invention relates to a new assay for the detection of phospho-*N*-acetylmuramyl-pentapeptide translocase enzyme activity (hereinafter referred to as "translocase enzyme").

Peptidoglycan is a major component of the bacterial cell wall that gives the wall its shape and strength. It is unique to bacteria and found in all bacteria, both gram-positive and gram-negative. Peptidoglycan is a polymer of glycan strands which are cross-linked through short peptide bridges. It consists of alternating β 1-4 linked residues of *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc). A pentapeptide chain is attached to MurNAc (MurNAc-pentapeptide) and the peptidoglycan polymers are crosslinked through these peptide chains.

Biosynthesis of peptidoglycan can be divided into three stages: firstly, synthesis of the precursors in the cytoplasm, secondly, transfer of the precursors to a lipid carrier molecule and, thirdly, insertion of the precursors into the cell wall and coupling to existing peptidoglycan.

Enzymes responsible for the biosynthesis of the peptidoglycan component of the bacterial cell wall are novel targets for the design of new antibiotics. Owing to the worldwide emergence of bacterial strains resistant to current antibiotics, it has become necessary to develop new antimicrobial agents. The translocase enzyme catalyses the first step in the membrane cycle of peptidoglycan biosynthesis, namely the transfer of phospho-*N*-acetylmuramyl-L-Ala- γ -D-Glu-*m*-diaminopimelic acid-D-Ala-D-Ala from Uridine 5'-diphosphate phospho-*N*-acetylmuramyl-L-Ala- γ -D-Glu-*m*-diaminopimelic acid-D-Ala-D-Ala (hereinafter referred to as "UDP-MurNAc-pentapeptide" or "UDP-MPP") to a membrane-bound lipid carrier, undecaprenyl phosphate. The translocase enzyme is encoded by the *mraY* gene in *Escherichia coli*.

The translocase enzyme is essential for bacterial viability (see D. Mengin-Lecreulx, L. Texier, M. Rousseau and J. Van Heijermoot, *J. Bacteriol.*, (1991), 173, 4625-4636).

5 No commercial antibiotics in current use are directed against the translocase enzyme. It therefore represents a target for novel antibacterial agents which has as yet been unexploited.

The translocase enzyme is usually assayed by radiolabelling the UDP-MurNAc-pentapeptide and monitoring the transfer of phospho-*N*-acetylmuramyl pentapeptide from the UDP-MurNAc-pentapeptide to undecaprenyl phosphate, resulting in the formation of a lipid intermediate, Lipid I. The radiolabelling is usually done either by using the enzyme Ligase to label the D-Alanine-D-Alanine end or by the *in vivo* incorporation on the membrane. Both these methods produce low yields and thus are not cost effective in developing high-throughput-screening (HTS) assays.

15 The translocase enzyme activity may alternatively be assayed using a fluorescent substrate such as dansyl chloride as described by Brandish *et al.*, *J. Biol. Chem.*, (1996), 271, 7609-7614. However, certain compounds may quench the fluorescence, thus resulting in picking up false inhibitors of the enzyme reaction.

20 It would be desirable to develop an assay for the translocase enzyme that is suitable for high-throughout screening.

In accordance with the present invention, there is therefore provided an assay for detecting phospho-*N*-acetylmuramyl-pentapeptide translocase enzyme activity, which comprises the steps of:

(1) incubating a reaction mixture comprising, in aqueous medium, *N*-succinimidyl [2,3-³H] propionate substituted UDP-MurNAc-pentapeptide, *N*-succinimidyl propionate substituted UDP-MurNAc-pentapeptide (non-radioactive), a source of divalent metal ions,

a source of undecaprenyl phosphate, a source of translocase enzyme and a detergent, under conditions suitable for enzyme activity to occur;

- (2) acidification of the reaction mixture with a suitable buffer comprising a quaternary ammonium salt at pH ~ 4.2 to stop the enzyme reaction of step (1); and
- (3) extraction of any undecaprenol-pyrophosphate-[2,3-³H]propionate-N-acetylmuramylpentapeptide product formed and measuring radioactivity using a scintillation counter.

In step (1), the UDP-MPP used may be any of those normally present in naturally occurring peptidoglycans. It is conveniently purified from bacteria or made enzymatically with precursors from bacteria, for example by methods similar to that described by Blaauwen *et al.*; J. Bacteriol. (1990), 172, 63-70. Alternatively, it may be isolated from cells of *B. subtilis* W23 by the methodology described by Lugtenberg *et al.*; J. Bacteriol. (1972), 109, 326-335. The preferred UDP-MPP to use is UDP-MurNAc-L-Alanine- γ -D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine from *Bacillus cereus*.

The UDP-MPP thus obtained is reacted with N-succinimidyl [2,3-³H]propionate (commercially available from Amersham Ltd.) to obtain N-succinimidyl [2,3-³H]propionate substituted UDP-MurNAc-pentapeptide (hereinafter referred to as "³H-propionated UDP-MPP").

The concentration of ³H-propionated UDP-MPP used in the assay will typically be in the range from 2 to 50 μ M, preferably from 2 to 40 μ M and more preferably from 2 to 25 μ M.

The concentration of the unlabelled, non-radioactive N-succinimidyl propionate substituted UDP-MPP (hereinafter referred to as "propionated non-radioactive UDP-MPP") also used in the reaction may be in the range from 5 to 70 μ M, preferably from 5 to 50 μ M and especially from 8 to 30 μ M.

Divalent metal ions used in the reaction are preferably magnesium ions. A suitable source of magnesium ions is magnesium chloride. The concentration of divalent metal ions used may be in the range from 20mM to 100mM, preferably from 20mM to 80mM, more preferably from 20mM to 50mM, e.g. 25mM.

In addition, potassium chloride at a concentration in the range from 50mM to 100mM may be added to the reaction mixture.

The membranes of *Escherichia coli* bacteria may conveniently be used and indeed are preferred as a source of undecaprenyl phosphate and translocase enzyme. The quantity of membranes used will typically be in the range from 5 to 200µg, preferably 50µg, per 50µl of the reaction mixture. The membranes may be prepared by methods known in the art.

The aqueous medium used in step (1) is preferably a buffer solution, e.g. of Tris [hydroxymethyl] aminomethane hydrochloride ("Tris-HCl"), having a pH of about 7.5. Tris-HCl is commercially available from the Sigma Aldrich Co. Ltd.

The reaction mixture may additionally contain 0.01 unit of alkaline phosphatase.

The detergent used may, for example, be Triton X-100 in a concentration of 0.1% w/v. The detergent may be effective in solubilising the bacterial membranes if these are used.

If the assay is intended to be used as a screen for identifying anti-bacterial compounds that are antagonists of the translocase enzyme, the reaction mixture in step (1) may further comprise one or more test compounds in varying concentrations. Since translocase is the enzyme required in the first step of peptidoglycan synthesis, it represents a suitable target for the development anti-bacterial drugs.

The reaction mixture of step (1) is maintained at a temperature in the range from 20°C to 37°C, preferably 25°C, for a short period of time, e.g. up to 10 minutes, specifically 6-8 minutes.

5 The enzyme reaction is stopped by the addition of, for example, 6M pyridinium acetate and n-butanol (pH ~ 4.2) in a 2:1 mixture. This constitutes step (2).

In step (3), the product is extracted using, for example, n-butanol. It is then quantified in a scintillation counter.

10 The present invention will be further illustrated with reference to the following Example.

Example 1

Eppendorf tubes were individually filled with a total of 40 µl of the reaction mixture each, initially. The reaction mixture consisted of an aqueous buffer solution of 100 mM Tris-HCl (Tris[hydroxymethyl]aminomethane hydrochloride), 25mM magnesium chloride, 50mM of potassium chloride, 0.1%w/v Triton X-100, 8µ M of propionated non-radioactive UDP-MPP, 2 µM of ³H-propionated UDP-MPP at room temperature, 10 µl of the enzyme at a concentration of 5 µg per ml and 17.5 µl of water. To this a solution of a test
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20 compound (e.g. Tunicamycin) of varying concentration is added. Tunicamycin is a known antagonist of the translocase enzyme. The reaction commences with the addition of the enzyme.

The *Escherichia coli* membranes, which serve as a source of the enzyme, were prepared in
25 the following manner.

The membranes are prepared from spheroplast pellets which are commercially available. Each pellet contains a certain amount of *E.coli* Hfr H. The pellet is thawed overnight at 4°C. The pellet is weighed and the figure is multiplied by 7.5. This gives the volume of
30 the buffer solution to be added to it. The buffer solution used is 20 µM Tris-HCl of pH

8.0, containing 20% sucrose. The mixture is stirred gently for 10 minutes using a magnetic stirrer in cold. To this is added, a solution of egg white lysozyme, till the concentration to get a final concentration of 0.2 mg per ml. It is stirred on ice for another 10 minutes. To this, EthyleneDiamineTetraacetic Acid (EDTA), commercially available from Sigma Aldrich Co. Ltd., of concentration 0.2 M in 20 mM Tris-HCl of pH 8.0, is added slowly over a period of one hour until a final concentration of 0.02M is obtained. This addition is carried out in a cold room at a temperature of 4-8°C. The mixture is then centrifuged at 12,000g for 20 minutes. The pellet is again resuspended in the same volume of 50 mM Tris-HCl buffer solution of pH 7.5 as calculated in the previous paragraph, containing, 20µg/ml DNase, 20µg/ml RNase, 1mM magnesium chloride and 1mM β-mercapto ethanol. This is stirred at room temperature for one hour until the sample becomes homogeneous. The membrane fraction is recovered by spinning in an ultra-centrifuge at 1,00,000g for an hour.

The ³H-propionated UDP-MPP used as the substrate is prepared in the following manner.

A fixed volume of 4 O.D. at a wavelength of 262 nm i.e. approximately 450-500 µg unlabeled UDP-MPP is taken in an empty eppendorf tube. In another eppendorf tube, 0.5mCi of tritiated N-succinimidyl propionate (N-succinimidyl [2,3-³H]propionate) is taken and to this, about 180µl of 1%NaHCO₃ is added. The second tube is mixed well and transferred to the first tube. The second tube is rinsed well, with 180µl of 1%NaHCO₃ and again transferred to the first tube. The tube, containing the mixture, is left overnight on a shaker at room temperature to facilitate labelling. In a similar manner, propionated non-radioactive UDP-MPP is prepared, using N-succinimidyl propionate instead of the radioactive compound.

It is purified as follows,

(1) A glass column is packed with 1ml of sephadex A-25, commercially available.

(2) The column is washed with a 10 bed volume of water.

(3) It is then equilibrated with a 10 bed volume of 1%NaHCO₃.

- (4) The reaction mixture is loaded onto the column and the flow through is collected.
- (5) The flow through is passed through the column twice, to facilitate binding.
- (6) The column is then washed with 0.5 ml of 1% NaHCO_3 .
- (7) The wash and flow through is collected in the same tube and labelled.
- (8) The column is washed with 6ml of 1% NaHCO_3 , twice.
- (9) The two washings are collected in the same tube and labelled.
- (10) The column is eluted with 1ml of 1% NaHCO_3 , containing 0.4M lithium chloride.
- (11) The fractions are collected and labelled.
- (12) The fractions are counted, and the purest ones used in the reaction.

The eppendorf tube containing the reaction mixture is incubated at 37°C for about 4 to 60 minutes and thereafter, every two minutes, $50\mu\text{l}$ of a 2:1 mixture of pyridinium acetate and n-butanol is added to stop the reaction.

- The product is extracted with saturated n-butanol and washed with $50\mu\text{l}$ of water. It is then counted, 25-50 μl at a time on a scintillation counter.

The reaction catalysed by the enzyme translocase is known to be reversible. To show the reversible reaction, the enzyme reaction was continued for 10 minutes to form the radioactive product, undecaprenol-pyrophosphate-[2,3- ^3H]propionate-N-acetylmuramylpentapeptide, Lipid I. This is separated from its organic phase. Once the radioactive product is formed one set of reactions is stopped using the 2:1 pyridinium acetate and n-butanol mixture as described before. In a parallel set of reactions, $1\mu\text{M}$ UMP is added. The reaction is then stopped after about 3-4 minutes. The lipid fraction can be extracted from both the sets of reactions. It is seen that radioactive count in the organic phase is reduced to basal levels with UMP. This indicates the reversal of Lipid I to the water soluble precursor.

This example goes to show that the present assay system is specific for only the translocase reaction, even when a particulate membrane was used as the enzyme source.

Figure 1 is a graph showing the counts per minute (cpm) versus time based on the readings taken from the 100% controls.

- 5 Figure 2 shows the rate of inhibition of translocase by Tunicamycin at 0.3 $\mu\text{g/ml}$ concentration (indicated by \blacktriangle) compared with the control (indicated by \blacksquare). This confirms that Tunicamycin is an antagonist of the translocase enzyme.

CLAIMS

1. An assay for detecting phospho-*N*-acetylmuramyl-pentapeptide translocase enzyme activity, which comprises the steps of:

(1) incubating a reaction mixture comprising, in aqueous medium, *N*-succinimidyl [2,3-³H] propionate substituted UDP-MurNAc-pentapeptide, *N*-succinimidyl propionate substituted UDP-MurNAc-pentapeptide (non-radioactive), a source of divalent metal ions, a source of undecaprenyl phosphate, a source of translocase enzyme and a detergent,

under conditions suitable for enzyme activity to occur;

(2) acidification of the reaction mixture with a suitable buffer comprising a quaternary ammonium salt at pH ~ 4.2 to stop the enzyme reaction of step (1); and

(3) extraction of any undecaprenol-pyrophosphate-[2,3-³H]propionate-*N*-acetylmuramylpentapeptide product formed and measuring radioactivity using a scintillation counter.

2. An assay according to claim 1, wherein the UDP-*N*-acetylmuramylpentapeptide which is substituted is UDP-MurNAc-L-Alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine.

3. An assay according to claim 1 or claim 2, wherein magnesium chloride is used as a source of divalent metal ions.

4. An assay according to any one of claims 1 to 3, wherein bacterial cell membranes are used as a source of one or both of undecaprenyl phosphate and translocase enzyme.

5. An assay according to claim 4, wherein the bacterial cell membranes are from *Escherichia coli*.

6. An assay according to any one of claims 1 to 5, wherein the reaction mixture of step (1) further comprises a test compound.

7. An assay according to claim 6, wherein the test compound is an antagonist of the translocase enzyme.

8. An assay according to any one of claims 1 to 7, wherein a 2:1 mixture of pyridinium acetate and n-butanol is used to stop the reaction in step (2).

9. An assay according to any one of the claims 1 to 7, wherein the product in step (3) is extracted using n-butanol.

10. A kit for use in performing an assay according to any one of the preceding claims, which comprises,

- (1) N-succinimidyl [2,3-³H] propionate substituted UDP-MurNAc-pentapeptide,
- (2) N-succinimidyl propionate substituted UDP-MurNAc-pentapeptide (non-radioactive),
- (3) a source of divalent metal ions,
- (4) a source of undecaprenyl phosphate,
- (5) a source of phospho-N-acetylmuramyl-pentapeptide translocase enzyme, and
- (6) a detergent.

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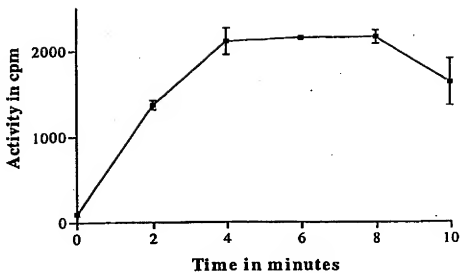


Fig. 1

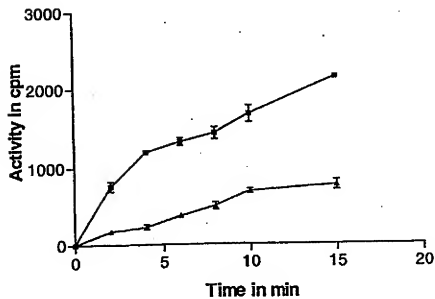


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/00772

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WD 0010587 A1 (INCARA PHARMACEUTICALS CORP.), 2 March 2000 (02.03.00), see page 4; and page 16, third paragraph ---	1-10
A	EP 0897007 A2 (SMITHKLINE BEECHAM CORPORATION ET AL), 17 February 1999 (17.02.99), see page 15, lines 41-55 ---	1-10
A	EP 0890644 A2 (SMITHKLINE BEECHAM CORPORATION), 13 January 1999 (13.01.99) ---	1-10
A	WD 9615258 A1 (THE UPJOHN COMPANY), 23 May 1996 (23.05.96) ---	1-10

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/00772

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Drug discovery today, Volume 1, No 7, 1996, Neil D. Cook, "Scintillation proximity assay: a versatile high-throughput screening technology" page 287 - page 294</p> <p style="text-align: center;">--- -----</p>	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

08/05/00

International application No.

PCT/SE 00/00772

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EP	0890644	A2	13/01/99	JP 11137248 A	25/05/99
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